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Anti-CD3/Anti-Epidermal Growth Factor Receptor-Bispecific Antibody Retargeting of Lymphocytes against Human Neoplastic Keratinocytes in an Autologous Organotypic Culture Model

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Local cellular immune defects have been described in several tumors including human papillomavirus (HPV)-associated cervical cancer. This observation suggests the potential therapeutic benefit of immune manipulations that restore cellular immunity. Here, we evaluated the ability of bispecific monoclonal antibodies (bimAbs) to redirect T cells against keratinocytes transformed *in vitro* by HPV in an autologous three-dimensional culture model (organotypic cultures). The epidermal growth factor receptor (EGFR) was chosen as target for an anti-CD3/anti-EGFR bimAb because it is overexpressed in many malignant epithelial lesions and only weakly expressed in the basal layers of normal squamous epithelium. Interestingly, in organotypic cultures, the pattern of expression of EGFR was similar to that observed *in vivo*. The ability of T cells retargeted by CD3/EGFR bimAb to lyse HPV-transformed cell lines was confirmed in monolayer cultures. In autologous organotypic cultures, an increase in apoptotic HPV$^+$ keratinocytes and a significant decrease in the thickness of HPV$^+$ organotypic cultures were observed when activated lymphocytes and bimAbs were added to the cultures, whereas organotypic cultures of normal keratinocytes were not significantly affected. These data were similar to those obtained in the allogeneic model. These results suggest the potential usefulness of CD3-EGFR bimAb-retargeted lymphocytes in immunotherapeutic protocols for malignant epithelial lesions. *(Am J Pathol 2002, 160:113–122)*

The cellular immune response against tumors is, in most cases, weak and mostly inefficient.[1] Bispecific monoclonal antibodies (bimAbs) have significant potential utility in human tumor immunotherapy as a tool for retargeting cytotoxic effector cells against tumor cells.[2] Indeed, cytotoxic lymphocytes can be recruited to kill tumor cells if they are retargeted by bimAb that binds both to the CD3 molecule associated with the T-cell receptor (TCR) complex and to a specific molecule expressed on the target cell surface.

Epidermal growth factor receptor (EGFR) is considered a suitable target molecule for antibody-driven immunotherapy because it is highly overexpressed in epithelial tumors.[3,4] EGFR overexpression was reported to maintain a proliferative pool of basal cells and to prevent the terminal differentiation of these cells in epidermal tumors.[5] An anti-CD3/anti-EGFR bimAb was generated and its efficacy in T cell retargeting has been documented in other tumor models *in vitro* and *in vivo*.[6]

Several parameters, such as cell-cell contacts and effector cell penetration of the tumors can dramatically affect the efficacy of bimAb-based immunotherapeutic protocols. Thus, preclinical *in vitro* and *in vivo* models that closely mimic the *in vivo* environment of the tissue of origin are prerequisite to validate new potentially therapeutic tools. Uterine cervical cancer is a good model to evaluate immunotherapy protocols, because the etiological agent of this tumor, the human papillomavirus (HPV), has been well-defined.[7,8] This cancer is preceded by well-characterized preneoplastic stages designated as squamous intraepithelial lesions. Moreover, several HPV proteins induce overexpression of EGFR by different mechanisms[9,10] and this overexpression is associated with poor prognosis in cervical carcinomas.[11]

The organotypic (raft) culture system has been increasingly used to examine the effects of viral or bio-

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chemical therapeutic agents on a variety of malignant keratinocytes.[12–15] The raft technique permits cell proliferation and differentiation at an air-liquid interface on a dermal equivalent support. Normal keratinocytes stratify and fully differentiate in a manner similar to the normal squamous epithelial tissues, whereas HPV-immortalized and established squamous carcinoma cell lines exhibit dysplastic morphologies similar to high-grade lesions seen *in vivo*.[16–18] Furthermore, we recently demonstrated that allogeneic activated lymphocytes are able to penetrate into raft cultures of squamous carcinoma cell lines.[15] To evaluate the retargeting ability of anti-CD3/anti-EGFR bimAb in a three-dimensional model and to avoid the potentially confounding effects of an allogeneic immune response, we set up a model with newly established HPV^+ cell lines and autologous effector cells. Our results indicate that bimAb-retargeted autologous lymphocytes induce apoptosis of autologous transformed keratinocytes, after migration into an *in vitro*-formed (pre) neoplastic epithelium.

Materials and Methods

Monoclonal Antibodies (mAbs)

The anti-CD3/anti-EGF-R bimAb M26.1 used in this study is secreted by a hybrid/hybridoma produced by somatic fusion of the mAb MINT5 (mouse IgG1), which specifically recognizes the EGFR, and the hybridoma producing mAb 298.1 (mouse IgG2a) that recognizes the human T-cell receptor-associated CD3 complex. Generation of the hybrid hybridoma and purification of bimAb have been described in detail.[19]

Culture of Keratinocytes

Normal human exocervical keratinocytes were isolated from hysterectomy specimens of women without disease related to the cervix. Cell cultures were established and maintained as described.[20] This study protocol was approved by the Ethics Committee of the Faculty of Medicine at the University of Liège.

SiHa and CasKi are cervical carcinoma cell lines containing, respectively, one copy[21] and ~600 copies of integrated HPV16 DNA.[22] C33 is a cervical carcinoma cell line negative for HPV.[23] KT1 and KT2 cell lines were established by transfecting the full-length HPV16 genome, linearized at the *Bam*HI site in pBR322-HPV16 plasmid, into short-term cultures of normal cervical keratinocytes. This transfection was performed by electroporation on 4.10^6 cells suspended in serum-free medium at 200 V and 950 μF using a gene pulser system (BioRad, Hercules, CA) as described previously.[20] These cell lines are tumorigenic in nude mice (unpublished observation). All cell lines were maintained in Ham F12 (1:3) mixed with Dulbecco's modified Eagle's medium (Life Technologies, Inc., Gaithersburg, MD), supplemented with 0.5 μg/ml hydrocortisone (Sigma, St. Louis, MO), 2 ng/ml EGF (Sigma), 10% fetal calf serum, 2 nmol/L L-glutamine, 1 μg/ml fungizone, 1 mmol/L sodium pyruvate, and 3000 U/ml penicillin-streptomycin (all from Life Technologies, Inc.).

For normal keratinocytes, we used the same medium supplemented with 10^{-10} mol/L cholera toxin, 5 μg/ml insulin, 20 μg/ml adenine, 5 μg/ml human transferrin, and 1.5 μg/ml 3,3′, 5-triiodo-L-thyronine (all from Sigma).

Isolation and Culture of T Lymphoblasts

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient (Ficoll-Hypaque) centrifugation from heparinized blood of healthy donors or hysterectomized patients, and cultured at 10^6 cells/ml with 50 IU/ml rIL-2 (Biosource, Nirelles, Belgium) and phytohemagglutinin (1 μg/ml; Difco, MI) or anti-CD3 (OKT3, 10 ng/ml). Culture medium was RPMI 1640 (Life Technologies, Inc.) supplemented with 5% pooled AB serum and 2 mmol/L L-glutamine (Life Technologies, Inc.). After 7 to 10 days of subculture, >95% of cells consisted of CD3+ T lymphoblasts.

Lymphocytes used for organotypic cultures were labeled with the lipophilic fluorescent marker CM-DiL (Molecular Probes, Leiden, The Netherlands) according to described procedures[24] with minor modifications. Briefly, lymphocytes were resuspended in 1 ml of phosphate-buffered saline (PBS) and warmed to 37°C. The stock solution of CM-DiL was diluted in 1 ml of PBS preheated to 37°C for a final concentration of 16 μg/ml. The dye was mixed and immediately transferred to the cell suspension. Cells were incubated for 2 minutes at 37°C followed by 2 minutes on ice, and washed in 40 ml of PBS at 4°C, centrifuged, and resuspended in the appropriate medium.

Cytotoxicity Assay

T lymphoblasts were used as effector cells in a 4-hour ^{51}Cr-release assay at effector-target cell ratios ranging from 50:1 to 5:1 with SiHa, CasKi, and C33, KT1, and KT2, and normal keratinocytes as targets. Briefly, 5×10^3 ^{51}Cr-labeled target cells were added to various numbers of effector cells in triplicate U-shaped 96-well microtiter plates. For the evaluation of bimAb-triggering, M26.1 bimAb was added at various dilutions (1 to 1000 ng/ml) in a final volume of 200 μl. Parental 289.1 (anti-CD3) or MINT-5 (anti-EGFR) mAb served as controls. After 4 hours of culture, 100 μl of supernatant were removed and evaluated for ^{51}Cr-release in a γ-counter. Percent lysis was calculated as described.[25]

Tumor Growth Inhibition Assay

Tumor growth inhibition was evaluated by a colorimetric assay with MTT (3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyl tetrazolium bromide) (Sigma) as described.[26] Target cells were seeded in triplicate at 7000/well for normal keratinocytes, 5000/well for KT1 and KT2 cells, 3000/well for C33 cells, and 2500/well for SiHa and CasKi cells, in flat-bottomed 96-well plates, with different numbers of effector cells and various mAb concentrations in 200 μl of

medium. After a 7-day incubation at 37°C, wells were washed twice to remove nonadherent cells (effector cells or dead target cells), and 100 μl of fresh medium containing 0.5 mg/ml MTT was added to each well. Cells were incubated for at least 4 hours at 37°C, and 100 μl of 2-propanol was added to each well, mixed thoroughly, and absorbance at 550 nm was determined in a microELISA reader (BioRad microplate reader 550; BioRad, Hercules CA). Percent growth inhibition was calculated as:

$$100 - \left(100 \times \frac{\text{A sample} - \text{A medium}}{\text{A control} - \text{A medium}}\right)$$

where controls represent target cells grown in medium alone. SD of triplicates never exceeded 5 to 10%.

Organotypic Cultures

Organotypic cultures of HPV-transformed and normal keratinocytes were prepared as described.[15] Briefly, dermal equivalents were produced using collagen I (ICN, Asse-Releigem, Belgium) containing 4×10^4 normal human fibroblasts. Then, 2.5×10^5 to 1×10^6 keratinocytes (HPV-transformed or normal) were seeded on top of the gels and kept submerged for ~24 hours. Rafts were then raised onto a stainless metal grid and cells allowed to grow at the air-liquid interface for 10 days. After stratification of keratinocytes, T lymphoblasts, preincubated for 30 minutes with or without 1 μg/ml M26.1 bimAb or parental mAbs, were seeded on top of the *in vitro*-formed epithelium at a concentration of 5×10^5 or 2×10^6 cells/50 μl of growth medium. After 48 hours at 37°C, collagen rafts were harvested. Cultures were embedded in OCT compound (Tissue Tek, Sakura, The Netherlands) at −70°C and sectioned with a cryostat microtome (Microm HM 5000 OM; Prosan, Merelbeke, Belgium) for fluorescent microscopic analysis (Olympus IX50, Micro Image 3.01.1 software).

Immunostaining

EGFR surface expression was evaluated on cells in monolayers using the MINT-5 mAb (1 μg/ml) followed by a secondary FITC-conjugated goat anti-mouse IgG (Immunotech, France). Incubation of primary and secondary antibodies was performed in PBS-bovine serum albumin (0.03%) for 30 minutes on ice followed by washes. Stained cells were analyzed on a FACScalibur sorter (Becton Dickinson, Erembodegem, Belgium) using CELLQuest software.

EGFR expression in biopsies of cervix and in organotypic cultures was assessed by immunohistochemistry with the avidin-biotin-peroxidase technique (Vectastain ABC Kit; Vector Laboratories, Burlingame, CA) using the anti-EGFR mAb MINT5. Frozen sections (6 μm) were fixed in cold acetone for 3 minutes and endogenous peroxidases were blocked with 0.1% H_2O_2 for 30 minutes. Sections were incubated sequentially with anti-EGFR antibody (1 μg/ml) or with isotype-matched control antibody for 1 hour, with a biotinylated mouse anti-Ig antibody for 30 minutes and with streptavidin/horseradish/peroxidase/avidin/biotin complex for another 30 minutes. Positive cells were visualized using 3,3′-diaminobenzidine substrate (Prosan). Sections were counterstained with methyl green.

Lymphocytes in organotypic cultures were immunohistochemically stained using an anti-CD45 mAb (DAKO, Belgium) followed by the same method as for EGFR staining.

Measurement of Organotypic Culture Thickness

Thickness of the organotypic culture was evaluated by the Micrometer program of the CAS "Cell Analysis Systems" (Becton Dickinson) and was expressed in μm. The complete section of the culture was screened and five measurements were obtained for each field.

Terminal dUTP Nick-End Labeling (TUNEL) Staining

Apoptotic cells were detected using the TUNEL technique (*In Situ* Cell Death Detection Kit; Roche, Germany). Briefly, slides were fixed in cold acetone for 3 minutes, washed twice with PBS, and 50 μl of TUNEL reaction mixture was added. After incubation in a humid chamber for 1 hour at 37°C, slides were washed three times with PBS, mounted, and examined by fluorescence microscopy (Olympus IX50, Micro Image software). Nuclei of all cells were revealed with DAPI staining (4′, 6-diamidine-2′-phenylindole dihydrochloride; Roche).

Enzyme-Linked Immunosorbent Assay (ELISA)

Levels of interferon-γ (IFN-γ) and tumor necrosis factor-α (TNF-α) in the cultures were measured using specific ELISA assays (Biosource, Nivelles, Belgium). Recombinant human IFN-γ and TNF-α were used as reference standards.

Statistical Analysis

The nonparametric Mann-Whitney test was applied using Instat Mac 2.01 software (GraphPad Software, San Diego, CA). Differences were considered significant at $P < 0.05$.

Results

Neoplastic HPV$^+$ Keratinocytes Overexpress EGFR

Fluorescence-activated cell sorting (FACS) analysis of EGFR on cell surface revealed high expression levels of EGFR on all HPV$^+$ keratinocytes (HPV-transformed keratinocytes KT1 and KT2 cells and tumor-derived SiHa and CasKi cells) whereas HPV$^-$ tumor cell line C33 showed expression level as low as that of normal keratinocytes (Figure 1A). EGFR was differentially expressed in the epithelium of the uterine cervix and, interestingly, this differential expression was also found in organotypic cul-

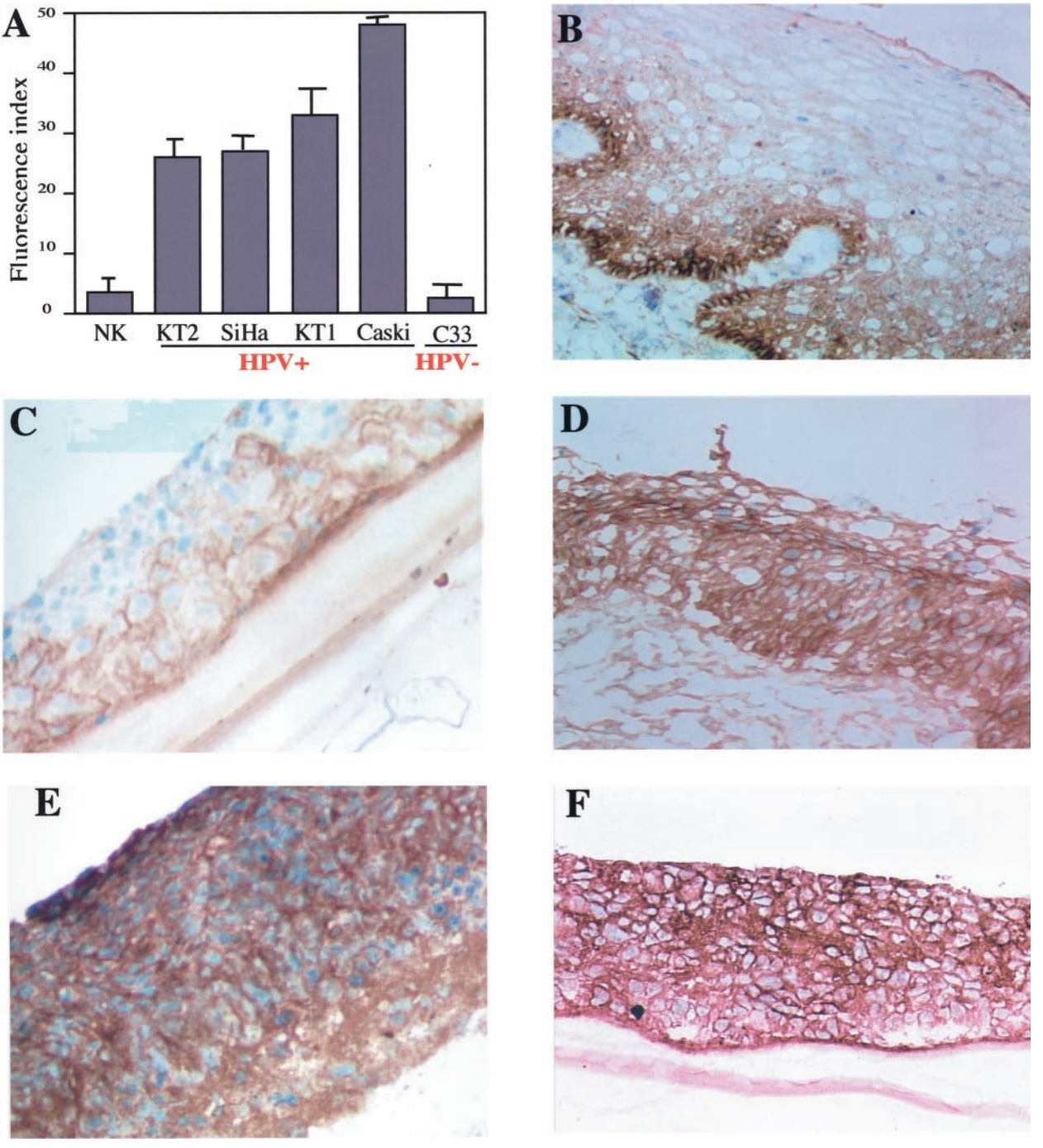

Figure 1. EGFR expression on normal and HPV$^+$ keratinocytes. **A:** EGFR expression by FACS analysis. Fluorescence index represents the total fluorescence intensity in the presence of mAb MINT5 and FITC-labeled secondary antibodies/background level in the presence of the FITC-labeled secondary antibody alone. Values are means (±SD) of five independent experiments. **B:** Immunohistochemical staining with mAb MINT5 on biopsy specimens (original magnification, ×20) of normal exocervix and high-grade squamous intraepithelial lesions (**D**), organotypic culture sections of normal keratinocytes (**C**), CasKi cells (**E**), and KT1 cells (**F**).

tures as indicated by immunohistochemistry staining. Indeed, staining was evident only in basal layers of normal exocervix biopsies (Figure 1B) and of normal keratinocyte organotypic cultures (Figure 1C), whereas all cells were strongly stained in high-grade cervical lesions (Figure 1D) and in organotypic cultures of HPV$^+$ cell lines CasKi, KT1 (Figure 1, E and F), KT2, and SiHa (data not shown).

Allogeneic Lymphocytes Retargeted by BimAb Kill HPV$^+$ Keratinocytes in Monolayer Cultures

To evaluate the efficacy of bimAb against HPV$^+$ keratinocytes, cytotoxicity assay of lymphocytes retargeted by the anti-CD3/anti-EGFR bimAb M26.1 was performed using normal and transfected cervical keratinocytes or cer-

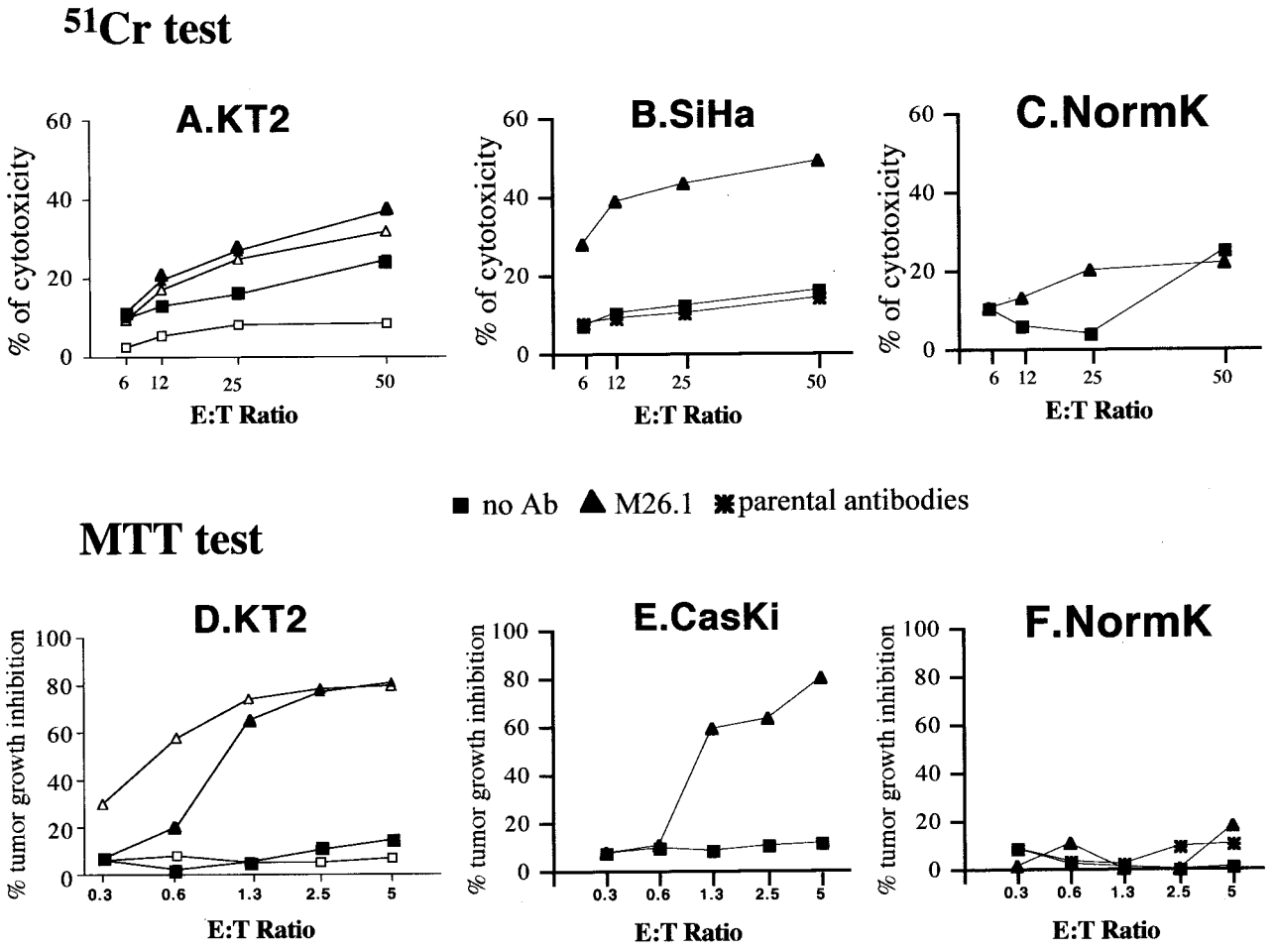

Figure 2. A–C: Cytotoxic activity of activated PBMCs in the presence or absence of anti-CD3/anti-EGFR bimAb M26.1 or of an equimolar mixture of the two parental antibodies in a 4-hour ^{51}Cr assay (only in **B**). **D–F:** Growth inhibition exerted by activated PBMCs in the presence or absence of anti-CD3/anti-EGFR bimAb M26.1 or the mixture of the two parental antibodies (only in **F**) in a MTT assay. Experiments were done in triplicate. **Lines with open symbols** in graphs **A** and **D** represent activity of autologous KT2 lymphocytes in the presence (**triangles**) or absence (**squares**) of M26.1. The other results were obtained with allogeneic PBMCs. Data are the means of replicates in one representative experiment. Similar results were obtained in two to five independent experiments with PBMCs from different healthy donors.

vical carcinoma cell lines in monolayer cultures as targets and lymphocytes from healthy donors as effectors. Cytotoxic assay revealed highly increased ^{51}Cr release in wells with activated T lymphocytes M26.1-retargeted and $EGFR^+$ target cells KT2, SiHa (Figure 2, A and B), and CasKi (data not shown) as compared to activated lymphocytes incubated in absence of bimAb, which exerted a low level of natural killer-like cytotoxic activity, particularly evident at the higher E:T ratios. Parental antibodies either alone or in combination failed to trigger cytolytic activity against SiHa cells (Figure 2B) or against the other targets (data not shown). A low but detectable cytolytic activity was also observed against normal keratinocytes (Figure 2C), which, as shown by FACS analysis, express only low levels of EGFR. A similar lytic activity was obtained against C33 HPV^- cells (data not shown), which express EGFR at levels comparable to those of normal keratinocytes.

In vivo, direct cytotoxicity is only one of the mechanisms that lymphocytes could use to kill tumor cells. Retargeted lymphocytes released cytokines able to inhibit the growth of tumor cells.[26,27] Thus, we tested this ability in a MTT tumor growth inhibition assay (Figure 2; D, E, and F). The inhibitory effect of retargeted lymphocytes on HPV-transformed keratinocytes KT2, CasKi (Figure 2, D and E), and SiHa (data not shown) grown as monolayers was much higher than that of lymphocytes alone, whereas growth of normal keratinocytes was unaffected at any E:T ratio tested (Figure 2F). Parental antibodies have no effects at all (Figure 2; D, E, and F).

Allogeneic Lymphocytes Retargeted by BimAb Kill HPV^+ Keratinocytes in Organotypic Cultures

Infiltration of allogeneic-activated lymphocytes redirected by bimAb M26.1 was evaluated in organotypic cultures of HPV^+ cell lines by staining the lymphocytes with an anti-CD45 mAb (results in Figure 3, A to D, shown for CasKi cells only). At both concentrations of lymphocytes tested (0.5 and 2×10^6 cells) infiltration by these cells was observed (shown in Figure 3B for the higher concentration). In the presence of activated lymphocytes and bimAb M26.1 (1 μg/ml), the thickness of CasKi or-

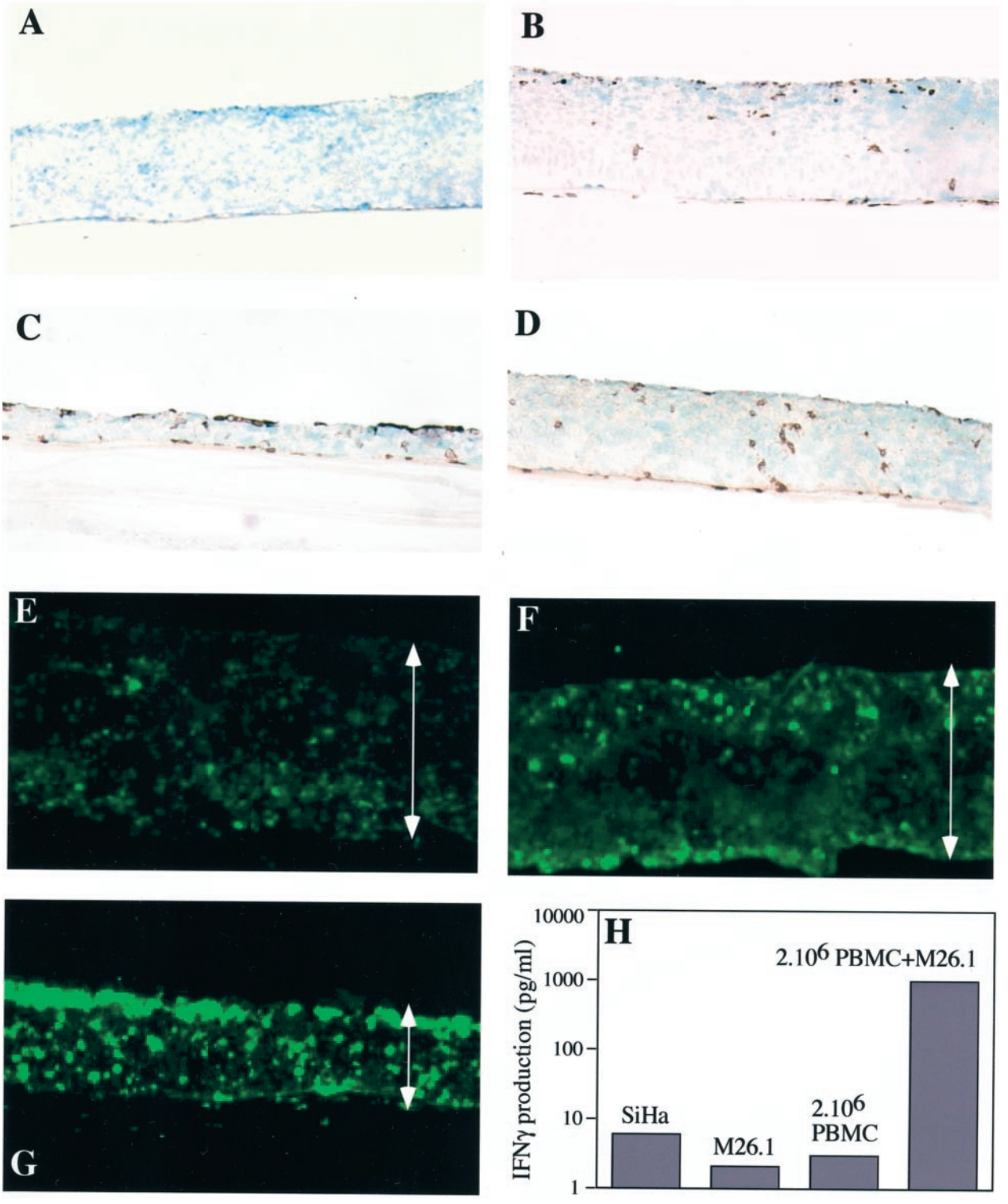

Figure 3. A–D: Infiltration and effects of allogeneic activated PBMCs on organotypic cultures of Caski cells (original magnifications, ×20). Lymphocytes were detected by staining with an anti-CD45 mAb. Cultures were incubated with medium only (**A**), with 2×10^6 PBMCs alone (**B**), with 2×10^6 PBMCs retargeted by bimAb M26.1 (**C**), or with 2×10^6 PBMCs incubated with anti-CD3 (298.1) and anti-EGFR (MINT5) mAbs at the same concentration as that of M26.1 bimAb (**D**). Similar results were obtained in three independent experiments with allogeneic PBMCs from different healthy donors. **E–G:** Detection of apoptosis by TUNEL assay in organotypic cultures of SiHa cells (original magnification, ×20) incubated with medium alone (**E**), with 1 μg/ml of bimAb M26.1 alone (**F**), or with 2×10^6 allogeneic PBMCs retargeted by bimAb M26.1 (**G**). **H:** IFN-γ production in medium of SiHa organotypic cultures as detected by ELISA assay.

ganotypic cultures was decreased (Figure 3C and Table 1) in correlation with the concentration of retargeted lymphocytes used (data not shown). In SiHa organotypic cultures, culture thickness in the presence of retargeted lymphocytes was also decreased as compared to SiHa cells alone (Table 1), but to a lower extent than for CasKi.

Table 1. Organotypic Culture Thickness

Target cells	*n*	Medium alone	2×10^6 PBMC	2×10^6 PBMC + M26.1	Thickness reduction, %‡
CasKi*	3	159 ± 2	128 ± 4§§	78 ± 3¶¶¶	38–51
SiHa*	3	319 ± 6	293 ± 7	212 ± 12¶	28–37
Normal keratinocytes*	3	165 ± 3	129 ± 2§§§	127 ± 4	15–21
KT1†	1	137 ± 2	119 ± 3	63 ± 2¶¶	55
KT2†	3	171 ± 3	140 ± 2§	97 ± 1¶¶¶	40–48

Data are given in μm (mean ± SE) from 1 to 3 independent experiments (*n*) with five separate measurements for each field.
*Target cells tested in the presence of allogeneic PBMCs.
†Target cells tested in the presence of autologous PBMCs.
‡Thickness reduction 2×10^6 bimAb-retargeted PBMCs *versus* medium alone.
§,¶*P* value < 0.05; §§,¶¶*P* value < 0.01; §§§,¶¶¶*P* value < 0.001. Thickness of organotypic cultures treated with bimAb-retargeted PBMCs *versus* PBMCs alone (¶), as well thickness of organotypic cultures with medium alone *versus* PBMCs alone (§) were compared. nd, not done.

Lymphocytes incubated with an equimolar mixture of parental anti-CD3 (298.1) mAb and anti-EGFR (MINT5) mAb showed no significant decrease in organotypic culture thickness (Figure 3D and Table 1).

The killing of HPV^+ keratinocytes induced by bimAb-retargeted lymphocytes was further examined by TUNEL assay to detect apoptotic keratinocytes. In SiHa organotypic cultures without retargeted lymphocytes, only a few apoptotic cells were present, probably because of spontaneous apoptosis (Figure 3E). In the presence of the bimAb M26.1 alone (Figure 3F) or nonretargeted allogeneic PBMCs (data not shown), the number of apoptotic cells was slightly increased. The addition of 2×10^6 lymphocytes and bimAb M26.1 significantly increased the number of apoptotic cells (Figure 3G). Similar effects were observed in CasKi organotypic cultures (data not shown). Addition of parental mAb (MINT5 and 298.1) to CasKi organotypic cultures led to a slight, but not significant, increase in apoptotic cell number (data not shown).

As already mentioned, retargeted lymphocytes might also release cytokines. Thus, we analyzed the production of IFN-γ and TNF-α in organotypic cultures of SiHa cells (Figure 3H) and CasKi cells (data not shown). IFN-γ production was high in both cultures in the presence of retargeted lymphocytes with 20- to 100-fold higher IFN-γ level with 2×10^6 retargeted lymphocytes than in absence of bimAb. Parental antibodies used as controls failed to induce IFN-γ production under the same experimental conditions. For example, in CasKi organotypic cultures, a production of 12 pg/ml with 2×10^6 PBMCs, 50 pg/ml with 2×10^6 PBMCs + parental mAb (MINT5/298.1), and 321 pg/ml with 2×10^6 PBMCs + M26.1 mAb were observed. Production of TNF-α in organotypic cultures of SiHa and CasKi cells was increased at least sevenfold in the presence of 2×10^6 retargeted allogeneic PBMCs relative to 2×10^6 PBMCs alone (data not shown).

To evaluate the possible side effects of lymphocytes retargeted by bimAb, organotypic cultures of normal HPV^- keratinocytes were also analyzed. These cultures incubated with lymphocytes retargeted by bimAb revealed poor penetration of lymphocytes, with no significant increase in the number of apoptotic cells. Both apoptosis and lymphocyte infiltration were limited to the superficial layers of the cultures (data not shown). A slight decrease in the thickness of the epithelial sheet was observed in these cultures in the presence of lymphocytes with or without bimAb (Table 1), probably because of allogeneic response of lymphocytes.

Autologous Lymphocytes Retargeted by BimAb Kill HPV^+ Keratinocytes in Organotypic Cultures

The ability of lymphocytes retargeted by M26.1 bimAb to lyse autologous HPV^+ keratinocytes growing in monolayer or in organotypic culture was tested using peripheral blood lymphocytes of patients from which the HPV^+ cell lines KT1 and KT2 were derived. The M26.1-retargeted KT2 lymphocytes had cytotoxic effects and inhibited the growth of the KT2 autologous cell line (Figure 2, A and D) and of CasKi cells (data not shown) at levels similar to those induced by healthy donors. In organotypic KT1cultures, the presence of autologous activated lymphocytes and bimAb led to a significant decrease in KT1 layer thickness (Figure 4 and Table 1) and this effect was proportional to the number of lymphocytes present. Consistent with the level of EGFR expression (see Figure 1A), the decreased thickness of the KT1 epithelial sheet was intermediate between that of Caski and SiHa cells (Table 1).

To identify the cell population (HPV^+ keratinocytes, lymphocytes, or both) undergoing apoptosis, lymphocytes were incubated with a red fluorescent lipophilic tracer before seeding on organotypic cultures of KT1. The majority of apoptotic cells were keratinocytes. Analysis of the number of apoptotic keratinocytes by TUNEL staining in the presence of medium (Figure 4A), 2×10^6 autologous lymphocytes (Figure 4C), or 2×10^6 M26.1-retargeted autologous lymphocytes (Figure 4E) revealed the highest apoptotic index with the bispecific antibody. These results were consistent with the thickness reduction (Figure 4, C and F, and Table 1). Superimposable results were obtained with KT2 cells in three separate experiments (Table 1). Moreover, IFN-γ (Figure 4B) and TNF-α (data not shown) production in the supernatant of KT1 and KT2 (data not shown) organotypic cultures were greatly increased in the presence of autologous lymphocytes retargeted by bimAb M26.1.

Discussion

The efficacy of immunotherapeutic protocols in preclinical studies is generally evaluated in allogeneic human *in*

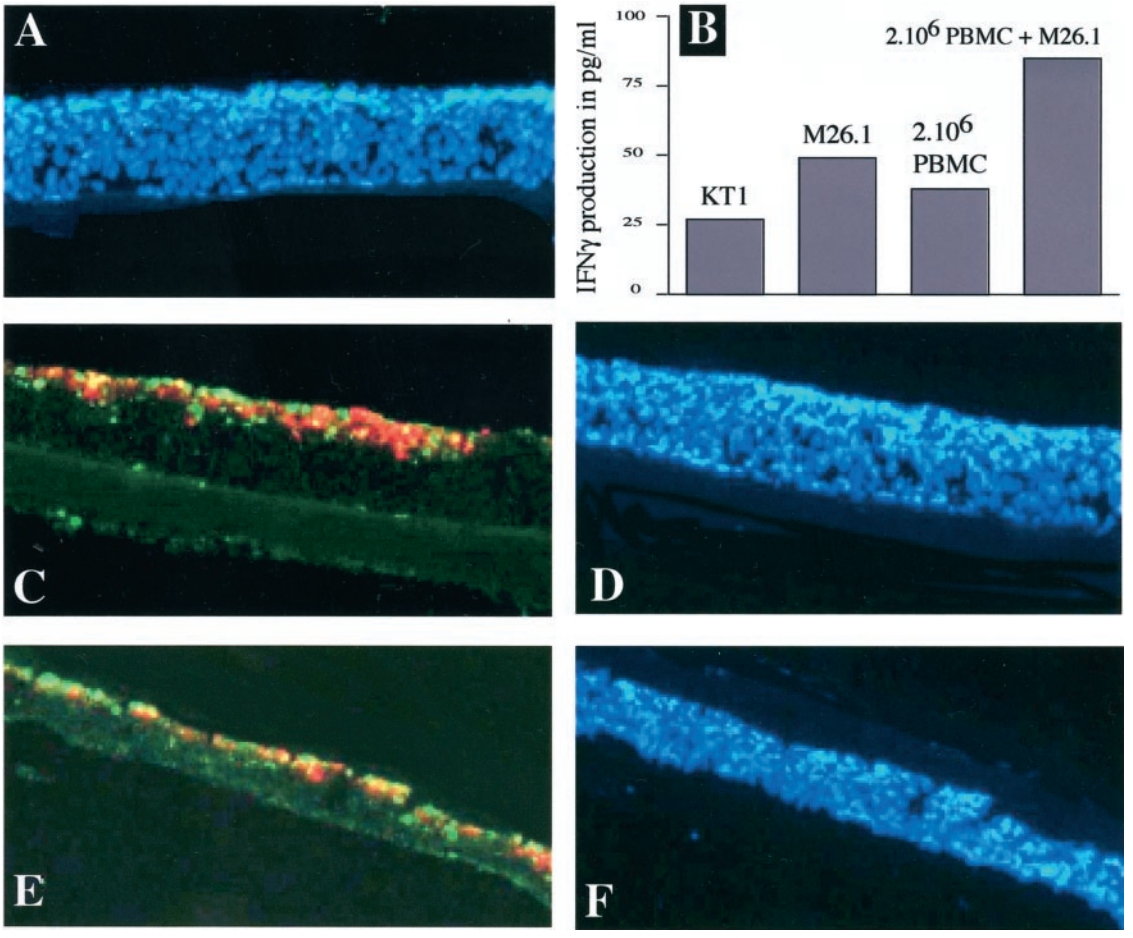

Figure 4. Detection of autologous lymphocyte infiltration and apoptosis in organotypic cultures of KT1 cells (original magnification, ×20). **C** and **E:** Staining of apoptotic cells by TUNEL assay (green cells) and infiltrating lymphocytes (red cells). **D** and **F:** The corresponding nuclei stained with DAPI. Organotypic KT1cultures were incubated with medium alone (**A,** TUNEL and DAPI staining are merged in this panel and very few apoptotic cells are present), 2×10^6 autologous PBMCs alone (**C** and **D**), or 2×10^6 autologous PBMCs retargeted by M26.1 bimAb (**E** and **F**). **B:** IFN-γ production in medium of KT1 organotypic cultures as detected by ELISA assay.

vitro models or in animals.[26,28–31] Here, we demonstrated the efficacy of lymphocytes redirected by a bimAb against neoplastic keratinocytes in an autologous three-dimensional culture model that closely mimics the *in vivo* situation. BimAb-redirected lymphocytes were able to reduce the thickness of autologous HPV$^+$ organotypic cultures and to induce keratinocyte apoptosis in these cultures.

The bimAb M26.1 recognizes the CD3 and EGFR molecules, a 170-kd transmembrane glycoprotein with tyrosine kinase activity. EGFR is overexpressed in a wide range of human malignancies[4,32,33] and increased EGFR expression is correlated with poor clinical outcome in patients with cancer of the lung, bladder, esophagus, breast, ovary, and cervix.[4] In light of the contrasting reports regarding EGFR up- and down-regulation[3,34,35] in cervical carcinogenesis,[11] we also analyzed EGFR expression in cervical preneoplastic lesions and demonstrated that EGFR expression in our model mimics the situation observed *in vivo*. Indeed, EGFR was expressed only in basal layers in normal exocervix, whereas all cells in high-grade cervical lesions highly overexpressed EGFR. These patterns were similar to those observed in organotypic cultures with normal keratinocytes or HPV$^+$ lines. FACS analysis confirmed EGFR overexpression in HPV$^+$ cell lines compared with normal keratinocytes and the HPV$^-$ cell line C33 and several mechanisms have been proposed to explain the EGFR overexpression by HPV.[9,10] The differential expression of EGFR on HPV$^+$ tumors might help to determine the bimAb concentrations that cause preferential damage to malignant cells.

Reduced cancer cell growth when the EGFR transduction pathway is abrogated has been reported for diverse therapeutic agents, including anti-EGFR monoclonal antibodies.[36] In our study, the parental anti-EGFR mAb (MINT-5) weakly inhibited the growth of HPV$^+$ keratinocytes in monolayer cultures. Similarly, in organotypic cultures of HPV$^+$ cell lines incubated with MINT-5 alone or with M26.1 bimAb alone, the number of apoptotic keratinocytes slightly increased but culture thickness was not reduced. This slightly increased apoptosis with M26.1 alone could be related to the blockage of EGFR, and indirectly to the inhibition of autocrine EGF or transforming growth factor-α activity. Accordingly Tosi and collaborators[6] have demonstrated that the F(ab)′2 fragment of MINT-5 (the anti-EGFR component of M26.1) inhibits

EGFR$^+$ tumor growth both *in vitro* and *in vivo* by interfering with transforming growth factor-α/EGF autocrine loops. Thus, the action of anti-EGFR mAb alone might be delayed compared with that of bimAb retargeted-lymphocytes and might appear after more than 48 hours of incubation. A major advantage of the bimAb-retargeting approach compared to the use of an anti-EGFR antibody alone is the induction of cytolysis and apoptosis of keratinocytes, resulting from the attack by bimAb-armed lymphocytes in addition to the blocking of the EGF pathway. Such tumor cell death provides a new source of antigens for the immune system, which might be relevant in light of recent evidence indicating that not only necrotic but also apoptotic cells can be captured by antigen-presenting cells and act as an antigen source for a specific T-cell response.[37,38]

Finally, bimAb-targeted T cells might also induce a local release of cytokines at the tumor site.[27] Despite the fact that cytokines production may differ donor to donor, we observed an increased production of IFN-γ and TNF-α in organotypic cultures of HPV$^+$ cell lines in the presence of retargeted lymphocytes. The cytokine levels were generally higher with allogeneic PBMCs. This difference could be because of an allo-MHC recognition that may synergize with bimAb effect and further enhance IFN-γ and TNF-α release. These two cytokines have pleiotropic biological activities, including anti-proliferative, anti-viral, and immunomodulatory effects.[39,40] Direct effects of these cytokines on the growth HPV-transformed keratinocytes in monolayer and in organotypic cultures have been reported.[13] Tumor growth inhibition induced by IFN-γ and TNF-α has been also demonstrated for other tumors.[41–43] Moreover, IFN-γ and TNF-α could initiate a local inflammatory response and indirectly attract antigen-presenting cells, which may be rare in cancers.[44] In particular, the presence of IFN-γ in the microenvironment could induce the T-helper 1 polarization of the T cell response that is associated with anti-tumor protection.[45] Furthermore, IFN-γ has been shown to enhance MHC class I molecule expression, which is frequently down-regulated in tumor cells.[46,47] IFN-γ is also known to up-regulate the expression of ICAM-1 on HPV-transformed keratinocytes,[48] which is important for the infiltration of lymphocytes into squamous lesions because stimulated lymphocytes express high levels of the ICAM-1 ligand LFA-1.[49] Up-regulation of ICAM-1 expression increases the susceptibility of tumor cells to bimAb-targeted lysis because the LFA-1/ICAM-1 cell adhesion pathway is involved in this mechanism.[50]

Human squamous epithelia express low to moderate levels of EGFR[35] raising the possibility of significant side-effects associated with the systemic use of anti-CD3/anti-EGFR antibody in humans. However, the F(ab′)$_2$ of this bispecific antibody can be used for local treatment at the site of the primitive tumor or for example, in serosal cavities. Some results have been already published about the *in vivo* anti-tumor efficacy of bimAb M26.1, eg, mean survival time of nude mice bearing human ovarian tumor xenografts was significantly increased after treatment with M26.1 bimAb-coated lymphocytes.[28] The potential immunogenicity of murine antibodies poses another problem in humans. However, advances in molecular genetics and protein engineering have led to new formats for recombinant bispecific antibodies, such as single-chain bispecifics, bispecific diabodies, and bispecific minibodies made from humanized or even fully human antibodies.[51]

In conclusion, our data demonstrate that a lymphocyte/M26.1 bimAb approach is efficient in killing autologous neoplastic keratinocytes in an *in vitro* model exhibiting many features of a squamous cell cancer on a mucosal surface. Together, the findings suggest the usefulness of bimAb in immunotherapeutic protocols for epithelial cancers.

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